

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 87303836.8

(51) Int. Cl.³: **C 08 B 37/10**

(22) Date of filing: 29.04.87

(30) Priority: 30.04.86 DK 1969/86

(43) Date of publication of application:
04.11.87 Bulletin 87/45

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: **NOVO INDUSTRIA/S**
Novo Allé
DK-2880 Bagsvaerd(DK)

(72) Inventor: **Nielsen, Jorgen Ilum**
Gedebakken 9
DK-3520 Farum(DK)

(72) Inventor: **Ostergard, Per Bjorn**
Rugvaenget 2
DK-4681 Herfølge(DK)

(74) Representative: **Brown, John David et al,**
FORRESTER & BOEHMERT Widenmayerstrasse 4/1
D-8000 München 22(DE)

(54) **Process for the preparation of LMW Heparin.**

(57) A method for production of low molecular weight heparin (LMW-heparin) by enzymatic batch depolymerization of heparin with heparinase wherein heparin is depolymerized to a desired weight average molecular weight by depolymerization to the corresponding number average molecular weight. The method enables depolymerization to a desired weight average molecular weight by measuring the increase in UV-absorption (preferably at 230-235 nm) ΔA_{235} and halting the depolymerization when ΔA_{235} has reached the calculated value corresponding to the desired number average molecular weight M_n .

The present invention relates to a method for the production of low molecular weight heparin (LMW-heparin) by enzymatic depolymerization of heparin.

Conventional heparin is a heterogenous mixture of mucopolysaccharides covering a molecular weight range from 5000 - 50000 daltons with a number average molecular weight of about 10 - 14000 daltons.

10 Heparin acts directly or indirectly on the function of a number of proteins particularly the enzymes of the coagulation cascade.

The effects of heparin are influenced by a number of factors, such as the distribution of functional groups in 15 the molecule and the molecular weight. Thus it is firmly established that the latter plays an important role for the activity of heparin, especially the inactivation of Thrombin and Factor Xa mediated by Antithrombin III.

Antithrombin activity demands a minimum heparin 20 molecular weight corresponding to about 18 monosaccharides i.e. about 5400 daltons whereas anti Factor Xa activity can be expressed by heparin molecules as small as 5-6 saccharide units 1500-1800 daltons.

A series of other effects of heparin e.g. 25 antithrombotic effect (heparin oligosaccharides containing 18 monosaccharides or less seem to have poor antithrombotic activity) influence on ADP-induced thrombocyte aggregation, bioavailability after s.c. administration, inhibition by PF₄ and HRG as well as the activity against coagulation enzymes 30 of the intrinsic pathway responsible for generating Factor Xa are strongly influenced by the molecular weight of heparin.

In recent years the interest has been centered on heparin fragments or - fractions with high XaI/ antithrombin activity with molecular weight from 4000 daltons to upwards 35 of 6000, since such substances have been reported to have good antithrombotic efficiency and at the same time no or

0244238

little tendency to cause bleeding complications. They also show enhanced bioavailability especially after subcutaneous administration.

Since the selectivity of heparin action is correlated to the molecular weight, it is likely that a relatively narrow molecular weight range exists in which heparin activity is optimal.

A method for preparing LMW-heparin with a specific, desired molecular weight and a narrow molecular weight distribution i.e. low polydispersity would therefore be advantageous.

The method of this invention enables attainment of any desired molecular weight range of depolymerization product from heparin.

LMW-heparin can be prepared in low yield from conventional heparin by fractionation (DOS 2,944,792 and 2,945,595). Most LMW-heparin is, however, prepared by depolymerization of heparin by either chemical or enzymatic methods followed by fractionation, if necessary (cfr. A. Horner, Heparin, Kakkar, eds. Thomas, 1976 and Perlin et al. Carbohydrate Res. 18, 185 (1971)).

Chemical depolymerization of heparin is described in EP published patent applications Nos. 0037,319, 0076,279 and 0014,184, US patent No. 4,351,938, and GB patent No. 2,002,406.

Enzymatic depolymerization is described in US patent No. 3,766,167, GB patent No. 2,002,406, EP published patent application No. 0014,184, and US patent No. 4,396,762.

A major problem inherent to all of the known batch depolymerization processes is to stop the depolymerization reaction at the correct average molecular weight. Moreover, the depolymerization reaction results in heparin fragments of smaller or larger size than the desired molecular weight, even in the absence of side reactions.

In the known depolymerization processes for depolymerization of heparin which use inorganic depolymerization reagents (nitrous acid, hydrogen peroxide,

0244236

etc.) no preference exists as to size of molecule attacked or as to position within the molecule of the bond to be broken. According to R.J. Linhardt et al., Biochem.Biophys.Acta 702 (1982) 197-203 not even the enzyme heparinase makes any such distinction the mode of action of heparinase being random endolytic.

This means that the polydispersity of any heparin depolymerization mixture develops in a statistically predictable way as a function of the degree of depolymerization. Especially at the time when average molecular weight is just above the desired value a large proportion of the fragments have the desired molecular weight but due to the random endolytic nature of the depolymerization they also have a proportionally large chance of becoming further depolymerized to give fragments of suboptimal size. A batch depolymerization should be halted at about this time.

However, heretofore the art has not developed satisfactory methods for controlling the depolymerization of heparin so as to obtain high yields of a predetermined LMW-heparin product.

An object of this invention is to provide a method for controlling an enzymatic depolymerization of heparin by heparinase in aqueous medium reaction mixture. It is a further object of the present invention to provide a method for the production of LMW-heparin with a desired average molecular weight.

Brief Statement of the Invention.

The present invention provides a process for the preparation of LMW-heparin by which heparin is partially depolymerized with heparinase in an aqueous medium, characterized in measuring the increase in light absorption during the course of such a depolymerization, the increase being caused by generation of an increasing proportion of

unsaturated heparin degradation products as the enzymatic depolymerization proceeds, and halting the enzymatic depolymerization when the increase in absorption has reached a value corresponding to the desired number average molecular weight M_n and the corresponding desired weight average molecular weight M_w , whereupon the LMW-heparin product is recovered from the reaction mixture.

The average molecular weight of a heparin depolymerization reaction mixture may be estimated in a number of ways based on e.g. GPC-HPLC, viscosity measurement, light scattering or chemical or physical-chemical determination of functional groups created in the depolymerization process.

Most of the methods mentioned such as GPC-HPLC are time consuming and not easily adapted to large scale production and in fact the majority of the known processes used in manufacture of LMW-heparin are based on empirical methods relying on careful control of starting composition and reaction conditions to obtain the desired molecular weight at the end of the depolymerization reaction. However, due to inevitable variations during the depolymerization reaction, e.g. variations in enzyme activity, the molecular weight of the end product may vary from one batch to another and making it difficult to obtain a uniform product in high yield.

If a product with the correct average molecular weight is to be produced by each production batch, the depolymerization reaction must be stopped immediately when the desired average molecular weight has been reached in the depolymerization reaction mixture. This requires that the change in the average molecular weight be followed by measurements for molecular weight determination having little or no lag time.

Heretofore no fast practical method existed for molecular weight determination of a depolymerization reaction mixture (containing LMW-heparin). One aspect of the present invention provides such a method.

The average molecular weight of heparin or a LMW-heparin may be given as the number average molecular weight (M_n), i.e. weight/number of moles, or as weight average molecular weight (M_w) or peak molecular weight (M_{peak}). M_w or M_{peak} are normally used to characterize heparin or LMW-heparin products.

It has now been confirmed experimentally that the polydispersity (D), i.e. M_w/M_n , of a given heparin depolymerization reaction mixture changes in a regular way during the enzymatic depolymerization reaction of heparin as shown in fig. 1.

The present invention is based on the fact that there has been developed a fast and reliable method to determine M_n during the depolymerization of heparin with heparinase. As fig. 1 provides the correlation between M_w and M_n the depolymerization of heparin to LMW-heparin of a given, desired M_w , can be achieved by depolymerization to a corresponding number average molecular weight M_n .

The enzymatic depolymerization process using heparinase lends itself to a spectrophotometric number average molweight (M_n) determination since the enzymatic process is eliminative creating one reducing endgroup and one endgroup consisting of a $\Delta 4,5$ -unsaturated-iduronic acid derivative having a distinct UV-absorption at 230-235 nm. The molar absorption coefficient for a number of LMW-heparin fragments of di-,tetra-,hexa-, and oligo saccharides was published by Linker and Hovingh (Biochem. 11(1972), 563-568). The average value of the published molar absorption coefficients is 5500.

30

$$\text{An equation such as } \frac{1}{M_n} = \frac{1}{M_{n,u}} + \frac{\Delta A_{235}}{c \cdot \epsilon} \quad (1)$$

giving the relationship between number average molecular weight (M_n) and increase in absorption at 235nm is easily derived.

In formula (1) M_n is the number average molecular weight of the depolymerized product, $M_{n,u}$ is the number average molecular weight of the heparin substrate, c is the substrate concentration (g/l), ΔA_{235} is the increase in absorption at 235 nm and ϵ is the molar absorption coefficient.

Calculation of M_n is possible when M_n of the heparin substrate ($M_{n,u}$), substrate concentration (c , g/l) and the absorption coefficient (ϵ) of the unsaturated depolymerization products are known and ΔA_{235} is measured.

10 In a number of experiments heparin was depolymerized with heparinase partially purified using hydroxyl apatite chromatography according to Linker and Hovingh (Methods in Enzymology 28(1972), 902-911).

The number average molecular weight M_n , was 15 calculated using equation (1) and using the published value of $\epsilon = 5500$, and compared to M_n determined by GPC-HPLC.

It was consistently found however that the calculated value of M_n ($M_n(\Delta A)$) differed from the value of M_n found using HPLC ($M_n(\text{HPLC})$), by up to 20%.

20 Rearrangement of (1) into

$$\Delta A_{235} = c \cdot \epsilon \left(\frac{1}{M_n} - \frac{1}{M_{n,u}} \right) \quad (2)$$

25 allows calculation of an absorption increase ΔA_{235} corresponding to a desired number average molecular weight M_n . But again experiments showed that if the depolymerization was halted at the calculated value of ΔA_{235} the actual M_n determined by HPLC was considerably higher than the desired 30 M_n if the value of $\epsilon = 5500$ found by Hovingh and Linker was used.

It was concluded that the poor correspondance between $M_n(\Delta A)$ and $M_n(\text{HPLC})$ was caused by the use of the value of $\epsilon = 5500$.

35 Rearrangement of equation (1) into

$$\xi = \frac{\Delta A_{235}}{c} \cdot \frac{M_n \cdot M_{n,u}}{(M_{n,u} - M_n)} \quad (3)$$

shows that ξ may be calculated using known values of c and $M_{n,u}$ and simultaneously determined values of ΔA_{235} and M_n (HPLC).

In this way a value of $\xi = 7600$ was found which gave close correlation between calculated $M_n(\Delta A)$ and observed M_n (HPLC) in a number of experiments.

10 The possibility of calculating a correct value of M_n based on the easily measured ΔA_{235} is basis for practice of the present invention according to which a batch enzymatic depolymerization of heparin is allowed to proceed until the calculated value of ΔA_{235} is reached whereupon the heparin
15 depolymerization reaction is halted and the reaction mixture is worked up.

The heparinase used according to the present invention is prepared in per se known manner as described by Hovingh and Linker (Methods in Enzymology 28 (1972), 902-911
20 and J.Biol.Chem. 245 (1970), 6170-6175) by culturing Flavobacterium heparium on a heparin containing substrate, cell harvesting and cell rupture by sonication and purification by among others chromatography on hydroxy-apatite. The degradation of heparin with heparinase is
25 conducted in an aqueous medium as described for instance by Hovingh and Linker (J.Biol.Chem. 240 (1965), 3724-3728). When the desired M_n value of the depolymerization mixture has been reached the heparinase is inactivated by known means, e.g. by lowering pH or a short heat treatment.

30 The LMW-heparin product is then precipitated by known means, e.g. precipitation with alcohol, and purified by methods well known in the art, e.g. bleaching, sterile filtration and alcohol precipitation.

Calculation of the increase in the absorption at
35 230-235nm ΔA_{235} corresponding to the desired M_w of the product is made as follows:

- a) reading on fig. 1 of M_n corresponding to the desired M_w and
b) calculation of ΔA_{235} corresponding to M_n from a) by means of the above formula (2) using the value 7600 for ϵ .

5 ΔA_{235} is measured by a spectrophotometer after acidification of the sample preferably to pH < 2.5. It is obvious for the person skilled in the art that the increase in UV-absorption caused by the formation of unsaturated
10 degradation products by the action of heparinase on heparin may be measured at other wave lengths than indicated here. The absorption coefficient is, however, preferably measured at 235nm because it has its maximum at this wave length.

The depolymerization is halted when ΔA_{235} has
15 reached the calculated value whereupon the LMW-heparin product is precipitated by addition of alcohol (preferably 0.6-10 vol/vol).

The heparin depolymerization reaction is preferably conducted at a temperature of 25-40°C and at a pH of 6-8.
20 Although the present invention is illustrated by means of a process in which heparinase is used in free form immobilized heparinase may also be used.

Example 1 illustrates the concordance of M_n as determined by A and ΔA measurements to HPLC measurements (M_n
25 (ΔA) to M_n (HPLC)) of samples throughout the course of a batch depolymerization of heparin.

Example 1

30 2.5 g heparin sodium, USP, $M_w = 17300$, $M_n = 12400$ daltons were dissolved in 25 ml 0.1 M sodium acetate, 0.01 M calcium acetate pH 7.0.

Heparinase, 0.8 ml, 1500 u/ml, specific activity 1050 u/mg was dissolved in 25 ml of 0.1 M sodium acetate.

35 One heparinase unit is defined according to Hovingh and Linker, Methods in Enzymol., 28(1972), 902-911.

The heparin substrate and the enzyme solution were mixed and incubated with gentle stirring in a water bath, thermostated at 30°C.

A sample taken immediately after mixing was diluted 5 with 1.7 M perchloric acid, pH less than 2.5, filtered and the absorption was measured at 235 nm.

The absorption measurement was repeated a number of times as shown in the table below and simultaneously taken samples were heated briefly on a boiling water bath to 10 destroy enzyme activity, cooled, diluted 5-fold with 0.5 M sodium sulphate and filtered and the molecular weight was analyzed by GPC-HPLC.

The GPC-HPLC analysis was carried out using Waters I-125 and I-60 columns in series with 0.5 M sodium sulphate 15 as eluent, 0.5 ml/min, monitored by refractive index detection, and the molecular weight was calculated according to retention time, using a non-linear standard curve based on dextran and heparin fragment standards.

The number average molecular weight, M_n , was 20 calculated from equation (1) using the absorption increase at 235nm, $\epsilon = 7600$, $c = 50$ and $M_{n,u} = 12400$.

The results are given in the table below and as seen, close agreement was obtained between the calculated and observed number average molecular weights.

25

Table I

	Time (hours)	A_{235}	ΔA_{235}	$M_n(\Delta A)$ (daltons)	$M_n(\text{HPLC})$ (daltons)	$M_n(\Delta A)/M_n(\text{HPLC})$ %
30	0	4.5	0	-	-	-
	1.0	28.83	24.33	6912	6750	102.4
	2.0	47.67	43.17	5148	4661	110.4
	3.0	63.43	58.93	4242	4261	99.6
	3.5	70.03	65.53	3951	3863	102.3
35	4.0	77.5	73.00	3666	3830	95.7
	4.5	83.2	78.7	3475	3565	97.5
	5.5	94.4	89.9	3153	3145	100.3
	22	242.5	238	1415	1405	100.7
						101.1

Example 2

The purpose of the experiments outlined below was to produce LMW-heparin of a weight average molecular weight 10 (M_w) of 6500 ± 500 dalton.

From figure 1 the number average molecular weight (M_n) corresponding to a $M_w = 6500$ dalton was found to be about 3500 dalton.

Five different heparins (see table below) were 15 chosen for enzymatic depolymerization. The change in optical density at 235 nm (ΔA_{235} (calculated)) corresponding to a M_n in the reaction mixture of 3500 dalton (in one experiment 3400 dalton) was calculated for each heparin from equation 2 using $c = 50$ mg/ml, $\epsilon = 7600$ and the $M_{n,u}$'s of the actual 20 heparin lots (see table).

The enzymatic degradation of the heparins was performed as follows:

Heparin was dissolved in a concentration of 50 mg/ml in 0.1 M sodium acetate buffer pH 7.0 containing traces 25 of calcium (0.0005 to 0.01 M). The solution was heated to 30°C and heparinase was added in the amount necessary to depolymerize the heparin to the desired M_n in about 48 hours.

The change in optical density at 235 nm (ΔA_{235}) was measured repeatedly in samples of reaction mixtures after 30 dilution with 1.7 M perchloric acid, pH below 2.5. When ΔA_{235} was equal to ΔA_{235} (calculated) the enzymatic depolymerization was halted.

When ΔA_{235} had reached the calculated value, the LMW-heparin product was precipitated by addition of alcohol 35 and the depolymerized product was purified by methods well

0244236

known in the art e.g. bleaching, sterile filtration and alcohol precipitation. Characteristics of the products from 5 independent experiments are shown in the following table.

Table II

Experiment No.	Batch size (g)	$M_{n,u}$ of heparin (dalton)	Desired M_n in product (dalton)	ΔA_{235} (calculated)	Amount of heparinase used (NOVO units/g heparin)	
10	1	1000	10100	3500	70.94	53
	2	1000	12400	3500	77.93	65
	3	1200	13900	3400	84.43	73
	4	1000	11600	3500	75.81	60
	5	1000	11200	3500	74.64	62

Experiment No.	Final ΔA_{235} in reaction mixture	Yield of product % (w/w)	M_n in product (dalton)	M_w in product (dalton)	Biological activity* of product IU/mg	
15	1	71.76	87.8	3690	6150	
	2	77.40	93.9	3840	6510	76
	3	83.97	90.6	3710	6630	77
	4	75.07	90.5	3660	6180	82
	5	74.80	93.0	3610	6610	82

* Biological activity by an amidolytic antifactor Xa assay using the 1. International LMW-heparin standard (National Institute for Biological Standards and Controls, London) as reference standard.

It appears from the above that all the final ⁰²⁴⁴²³⁶LMW heparin products have a weight average molecular weight (M_w) within the desired range.

The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.



5

CLAIMS

10

1. A process for the production of LMW-heparin by which heparin is partially depolymerized with heparinase in an aqueous medium characterized in that the increase in light absorption caused by the creation of unsaturated degradation LMW-products by the action of heparinase on heparin is measured during the depolymerization reaction, the reaction is halted when the increase in absorption has reached a value corresponding to the desired number average molecular weight M_n and the corresponding weight average molecular M_w weight and LMW-heparin is recovered from the reaction mixture.

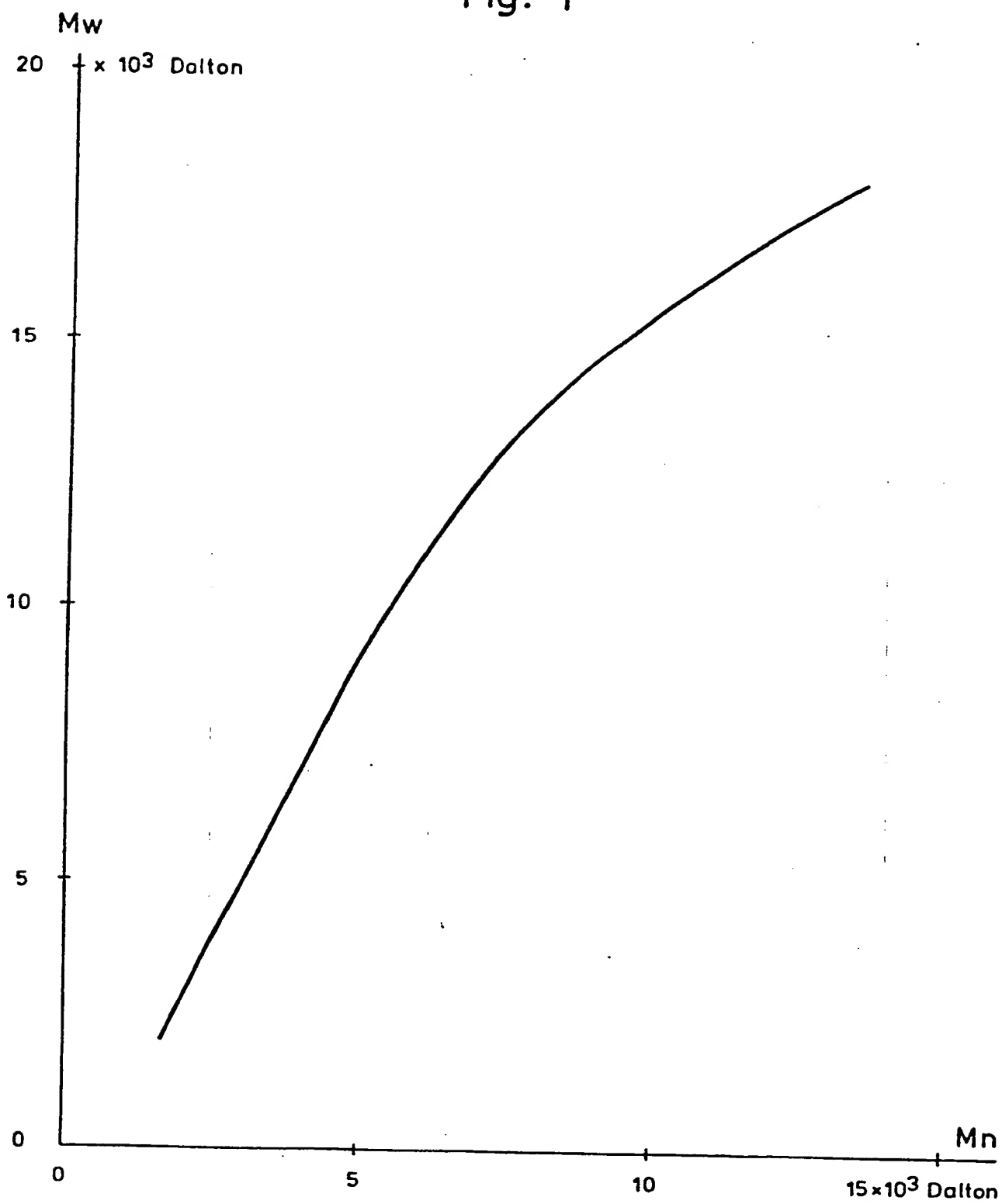
2. A process according to claim 1, wherein the increase in absorption coefficient is measured at 230-235 nm.

3. A process according to claim 1, wherein the heparinase is used in free form.

30

0244236

Fig. 1



(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 244 236
A3

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 87303836.8

(51) Int. Cl.: C 08 B 37/10

(22) Date of filing: 29.04.87

(30) Priority: 30.04.86 DK 1969/86

(43) Date of publication of application: 04.11.87
Bulletin 87/45

(84) Designated Contracting States: AT BE CH DE ES FR GB
GR IT LI LU NL SE

(88) Date of deferred publication of search
report: 28.09.88 Bulletin 88/39

(71) Applicant: NOVO INDUSTRI A/S, Novo Allé,
DK-2880 Bagsvaerd (DK)

(72) Inventor: Nielsen, Jorgen Ilum, Gedebakken 9,
DK-3520 Farum (DK)
Inventor: Ostergaard, Per Bjorn, Rugvaenget 2,
DK-4681 Herfølge (DK)

(74) Representative: Brown, John David et al, FORRESTER &
BOEHMERT Widenmayerstrasse 4/1,
D-8000 München 22 (DE)

(54) Process for the preparation of LMW Heparin.

(57) A method for production of low molecular weight heparin (LMW-heparin) by enzymatic batch depolymerization of heparin with heparinase wherein heparin is depolymerized to a desired weight average molecular weight by depolymerization to the corresponding number average molecular weight. The method enables depolymerization to a desired weight average molecular weight by measuring the increase in UV-absorption (preferably at 230-235 nm) ΔA_{235} and halting the depolymerization when ΔA_{235} has reached the calculated value corresponding to the desired number average molecular weight M_n .

EP 0 244 236 A3



European Patent
Office

EUROPEAN SEARCH REPORT

0244236

Application Number

EP 87 30 3836

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D,A	US-A-4 396 762 (R.S. LANGER et al.) * Claim 1 *	1	C 08 B 37/10
A	WO-A-8 103 276 (RIKER LABORATORIES) * Pages 5,13,14 * & US-A-4 351 938 (Cat. D)	1	
D,A	BIOCHEMISTRY, vol. 11, March/April 1972, American Chemical Society, Washington, D.C., US; A. LINKER et al.: "Isolation and characterization of oligosaccharides obtained from heparin by the action of heparinase" * Figure 2; page 565, lines 1-3 *	1	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
			C 08 B
Place of search THE HAGUE		Date of completion of the search 11-07-1988	Examiner SOMERVILLE F.M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	